American Fern Journal 101(3):182–192 (2011)

An Efficient Regeneration Pattern via Gemmae for *Huperzia serrata* (Thunb. ex Murray) Trev. in Hainan Province, China

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Abstract.—In the present study, we investigated a natural population of *H. serrata* in the Bawangling Nature Reserve of Hainan Province, South China. In field sampling, we examined the number of adult plants, gemmlings and sporelings as well as the gemma number of plants at different ages. A significant difference was observed between the numbers of gemmlings and sporelings. Most seedlings derived from gemmae, which critically influenced the population regeneration. The reproductive ability of gemmae became stronger from the 4th year of gemma growth. In the cultivation test, gemmae were planted in three different soil media, i.e., habitat soil, sand and humus. No significant difference was found in the gemmation rate among the three media, but the survival rate in sand was significantly lower than in the other two media. We also investigated the morphology of the gemma and gemmling growth pattern of *H. serrata*. The results may reveal the contributing role of gemmae in reproductive strategies, and be helpful to the resource protection and cultivation of *H. serrata*.

Key Words.—*Huperzia serrata* (Thunb. ex Murray) Trevis., Gemma, Gemmling, Sporeling, Regeneration pattern

Huperzia serrata (Thunb.ex Murray) Trevis. (formerly *Lycopodium serratum* Trevis., Huperziaceae) is a club moss, and commonly named Qiancengta in Chinese. *Huperzia serrata*, a perennial evergreen herb of 10–30 cm in height, grows well in forest, especially in shadowy, wet and humus-rich habitats. It is widely distributed from temperate East Asia to tropical Southeast Asia, even through Pacific islands to Central America. In China, this plant is distributed throughout the southern area (Ching, 1981; Zhang *et al.*, 2000). Since Liu *et al.* (1986 a, b) first extracted and isolated Huperzine A (Hup A) from *H. serrata*, this plant has attracted great attention. As a potent, reversible and selective acetylcholinesterase inhibitor capable of improving memory ability, Hup A is used in the treatment of Alzheimer's disease (AD) (Tang *et al.*, 1996). However, the great medicinal value of Hup A has caused its over-exploitation in recent years, and has greatly depleted stored material (Ma *et al.*, 2006).

The spatial distribution patterns of plant populations are determined by processes of growth, mortality and regeneration of individuals within the

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population (Ehrlen and Eriksson, 2000). Although *H. serrata* is distributed widely in temperate and tropical areas, these populations are usually small and scattered (Wu *et al.*, 2005). Its population size and structure are generally restricted to its propagation pattern and ecological factors. Its gametophyte and sporophyte usually grow slowly in habitats with relatively high humidity and rich organic matter. Spore propagation in other species of *Huperzia,* and the related genus, *Lycopodium,* has long been studied by botanists (Primack, 1973; Whittier *et al.,* 1986, 2006, 2007), but little is known about the process of spore germination and the morphology of gametophyte of *H. serrata.* Besides the spores and shoot culture, the specific vegetative propagules, which are called gemmae or bulbils, play an important role in dispersal and regeneration of *Huperzia* (Øllgaard, 1987; Reutter, 1987; Gola, 2008). Wang *et al.* (2007) studied the gemma morphology and seedlings of *H. javanica* (Sw.) Fraser-Jenk., a species closely related species to *H. serrata,* which can also produce gemmae. In Hainan populations, the adult plants produce new leaves, sporangia and gemmae from February to March, and usually the sporangia crack open to release spores in December and the following January. The gemmae fall down during the period from August to October. However, whether gemmae or spores are the main regeneration process of the natural population in Hainan Province remains unknown.

A lack of knowledge on the lifecycle and population structure of *H. serrata* results in difficulty in cultivating and propagating this plant. To solve the above problem, we investigated the natural population of *H. serrata* in the Bawangling Nature Reserve of Hainan Province from 2008 to 2009. We focused on the potential value of gemmae for propagation during the survey. Meanwhile, we observed the gemma structure of this plant both in field and in lab. Some mature gemmae were brought back to the botanical garden of our institute for cultivation tests in different soil media. Here we report the natural population structure, the gemma morphology and the survival rate of gemmlings of *H. serrata* in different media.

MATERIALS AND METHODS

Study site.—This study took place in the tropical montane evergreen dwarf forest (alt.1300 m) in the Bawangling Nature Reserve of Hainan Province, South China. The climate is tropical monsoon, with annual precipitation of 1750 mm. Most of the precipitation occurs between May and October (Zang *et al.*, 2005). The parent material is granite and the soil is latosols (Lu *et al.*, 1986). The main concomitant species in the arbor layer include *Lithocarpus howii, Ternstroemia gymnaanthera, Symplocos chunii, Rhododendron klossii, R. moulmeinense,* and *Pinus fenzeliana.* The herbanceous layer consists of some species of ferns and mosses, such as *Abacopteris* sp., *Selaginella* sp., *Dumortiera* sp., *Frullania* sp. and *Radula* sp.

Materials.—The voucher (Zhu2008001) was deposited in the Herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical

Sciences (IMD). The gemmae for the cultivation test were all collected from this population.

Investigation methods in field.—We investigated for the first time the natural population of *H. serrata* in August, 2008. Since then we have continually observed the development of the population and we collected experimental materials more than ten times in other seasons.

In the field investigation, 36 quadrates were randomly set up (0.25 m^2 each quadrate) in a 10 m $\times$ 10 m area. The investigated items for each quadrat included plant number, seedling (including the gemmlings and sporelings) number, plant height, and gemma number.

Observation of gemma and cultivation experiments.—Mature gemmae were brought back for observation of gemma structure and cultivation experiment. Observation and photography of dissected gemmae were conducted with a light microscope (Nikon SMZ1500). A drawing of an adult sporophyte was made to explain the relationship between the gemma layer and the morphology of the plant.

Mature gemmae were collected from the above-mentioned natural population of *H. serrata* and cultivated in a sunlight-proof shed in December, 2008. Although gemmae shed from August to October, the gemmae survived for 2–3 months at 4°C after they were collected. The media adopted were as follows: the habitat soil (from the 5 cm topsoil of the *H. serrata* habitat, which contained the humus of mosses and leaves of conifers, fagaceous and theaceous trees), sand (from the Medicinal Botanic Garden of the Hainan Branch of the Institute of Medicinal Plant Development), and humus (from the 5 cm surface plantation soil of the above garden). For each treatment, we gathered enough soil for 3 repeats, mixed it well and put it into pots. The pots, 16 cm in diameter and 20 cm in depth, were placed in a sunlight-proof shed with 20% transmittance. The gemma bases were buried downward in the medium, at the depth of 2/3 gemma length and 20 gemmae per pot. The gemmation rate was calculated after 30 days cultivation, and the gemmling survival rate was calculated after 120 days cultivation. The cultivation was carried out at temperature of 18–30 °C and humidity of above 80%. From January to April in 2009, the gemmation rate of gemmae and the survival rate of gemmlings in different media were recorded. With the above planting method, we specifically planted some gemmae in other pots for the physiological observation of gemmlings. The observation procedure is as follows: we pulled the gemmae from the soil and cleaned them with flowing water. Then we observed the growth of young roots and shoots, and replanted them after observation. We pulled out different gemmae each time.

Data collection.—All plants sampled in the natural population were divided into three groups. Group1 (G1): the plants with gemmae or sporangia; Group 2 (G2): the plants without gemmae or sporangia, but with remnants of gemmae and modified leaves at the stem base; Group 3 (G3): the plants without gemmae and sporangia, but with modified leaves at the stem base. The plants in G1 were considered as adult plants, while those in G2 and G3 covered all young plants, gemmlings and sporelings.

In natural populations, most plants produce gemmae and gemmiphores,once a year. The gemmae shed from mother plants in the same year after gemmae come into being, but the gemmiphores remain on stems for several years, even until plant death. Accordingly, we divided the adult plants into several groups in order of gemmiphore layers. The adult plants with gemmae were subdivided into six groups according to their ages, i.e. G_{n+1}, G_{n+2}, ..., G_{n+6} , because gemmae usually form one layer annually. Here n represents the growth years before gemmae appeared, and 1–6 indicate the layer numbers of each plant, respectively. The sixth group included the plants with 6 layers and >6 layers, which is very rare in all plants.

Statistical analyses.—We used one-way ANOVA to examine the main pattern of population regeneration, the gemma number per plant per year and the more suitable matrix to cultivate gemmae.

We counted the number of sporlings and gemmlings in the natural population and calculated their respective proportion out of the total number of plants. Then we compared the difference between sporelings and gemmlings proportions by one-way ANOVA to make clear the main regeneration pattern of this population. We also counted the gemma number per year in those plants with different gemma layers. We tested and compared the difference between the gemma numbers by one-way ANOVA and Least Significant Difference (LSD) to determine the changing trends of gemma number along with the increase of gemma layers in those plants at different ages.

In the cultivation experiment, we calculated the gemmation rate of gemmae and the survival rate of gemmlings in different cultivation media. The gemmation rate and survival rate are calculated as follows:

$$Rg = ng/Nt \times 100\%,$$

$$Rs = ns/Nt \times 100\%,$$

Where Rg is the gemmation rate of gemmae, Rs is the survival rate of gemmlings, ng is the gemmation number after 30 days cultivation, Rs is the gemmling number after 120 days cultivation, and Nt is the total number of cultivated gemmae. By homogeneity of variance and LSD, we analyzed the difference between different treatments to choose the better media for gemma cultivation.

Results

Gross morphology of H. serrata.—Fig. 1 shows an adult plant of *H. serrata* with three grades (I, II, and III) of branches. The plant is dichotomous with the small leaves (sl) and the big leaves (bl) arranged alternatively along the branches. Sporangia (s) exist in axillae of most leaves, and gemmae (ge) or gemmiphores (gp) appear above every layer of big leaves. Gemmae are generally present from February to March, and fall down from August to

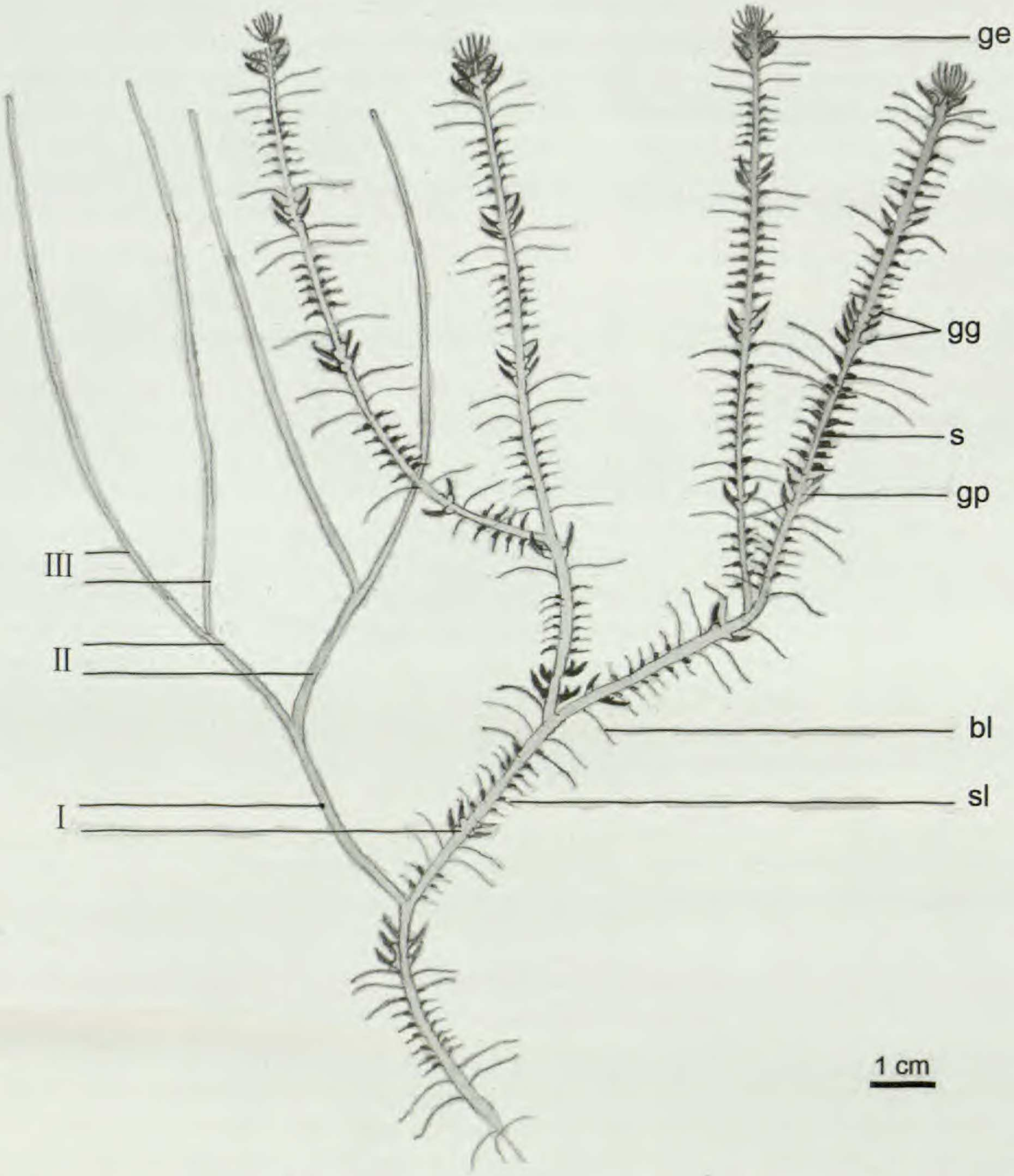

FIG. 1. The pattern figure of *H. serrata* sporophyte. (I) The first branch; (II) The second branch; (III) The third branch. sl: small leaf; bl: big leaf; s: sporangium; gp: gemmiphore; gg: a grade of gemmiphore; ge: gemma on the gemmiphore. Scale bar =1 cm.

October, usually once a year. The layers of gemmae or gemmiphores may be used as indicators to judge the plant ages.

Structure and number of gemmae.—As shown in Fig. 2, the gemmiphore with five modified leaves encloses a gemma (Fig. 2A, E). The gemma with the dorsal-ventral pattern is composed of one bud and six gemma leaves (Fig. 2A,

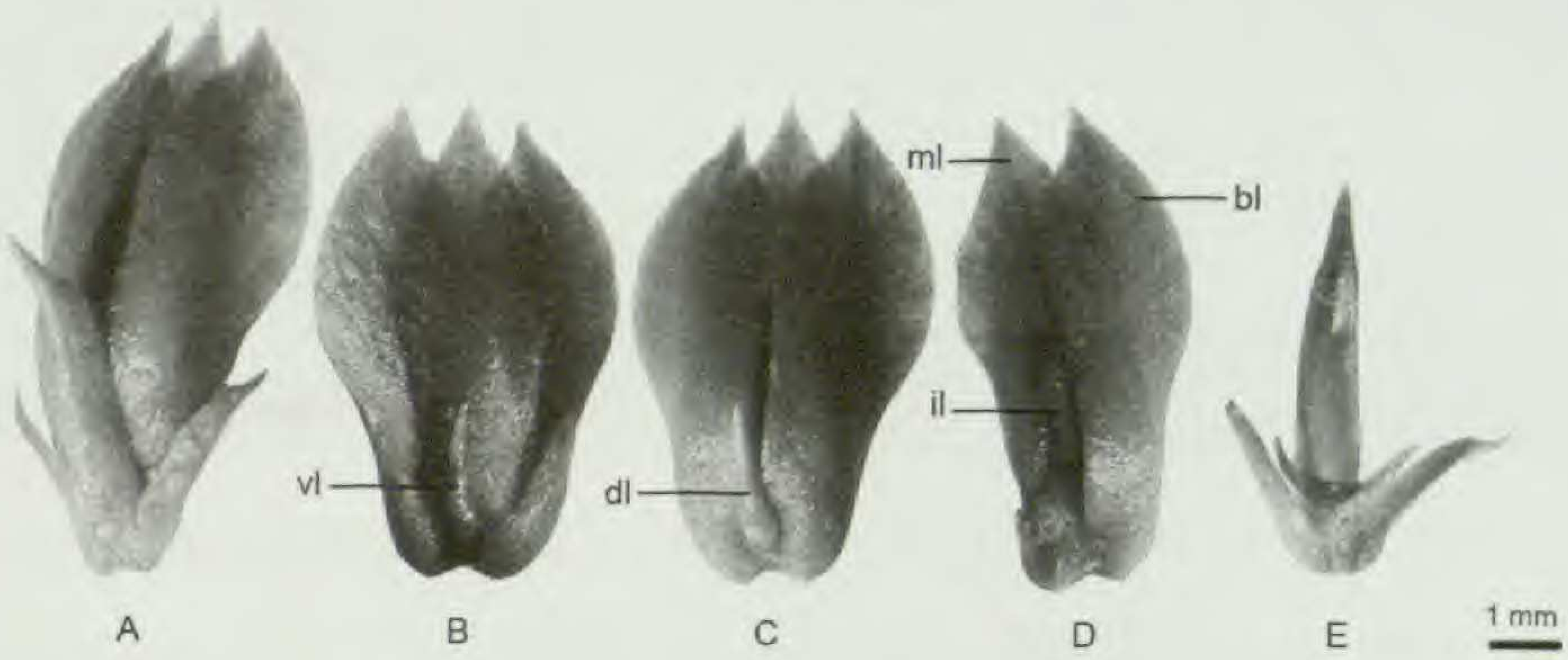

FIG. 2. Gemmiphore and the gemma structure. (A) Gemma and gemmiphore; (B) ventral view of the gemma (vl: ventral small leaf of the gemma); (C) dorsal view of the gemma (dl: dorsal small leaf of gemma); (D) leaves of the gemma (il: inner gemma leaf, bl: big gemma leaf; ml: middle big gemma leaf)(dorsal view); (E) gemmiphore. Scale bar (A, B, C, D, E)=1 mm.

B, C, D). These structures are succulent and dark green. Three big gemma leaves and one small gemma leaf are on the dorsal side (Fig. 2C), and the small one is on the ventral side (Fig. 2B). The bud is on the inner side of the middle gemma leaves (ml and il) (Fig. 2D). This structure is similar to that of a *H. javanica* gemma (Wang *et al.*, 2007).

Gemmae usually form one layer annually, and each layer includes one to several dozens of gemmae. The mean gemma number of G_{n+1} was 6.3. The number usually increased along with plant ages, reaching 30.0 in G_{n+4}, and then decreased slightly in G_{n+5} and G_{n+6} (Fig. 3). One-way ANOVA analysis showed that there was significant difference between G_{n+2} and G_{n+3} ($p<0.01$). The average gemma number was over 20 for the plants with more than three layers.

Population structure.—The investigated population covered a total area of 150 m^2. Most plants concentrated at a high density in over 100 m^2. Most seedlings derived from the fall-off gemmae in this population. Green, light-yellow or colorless gemma leaves remained at the base of the seedling stems for more than one year, which was considered as the evidence for the plants derived from gemmae.

We sampled a total of 605 plants in 36 quadrats, including 80 adult plants (G1) all producing gemmae, 315 plants (G2) developed from gemmae and 210 plants (G3) from spores. Those gemmlings accounted for 60% of the total of young plants, sporlings and gemmlings, while the sporelings accounted for 40% (Fig. 4). Significant difference was observed between gemmlings and sporelings ($p<0.01$).

The gemmation rate and survival rate of gemmae under cultivation conditions.—The cultivated gemmae germinated accompanied by splaying of surrounding modified leaves after 3 or 4 days; at the same time, very short roots ($\approx$ 0.1 mm) appeared at the base of gemmae. Seven days later, gemmlings grew out one or two entire leaves, which were narrow and bright green.

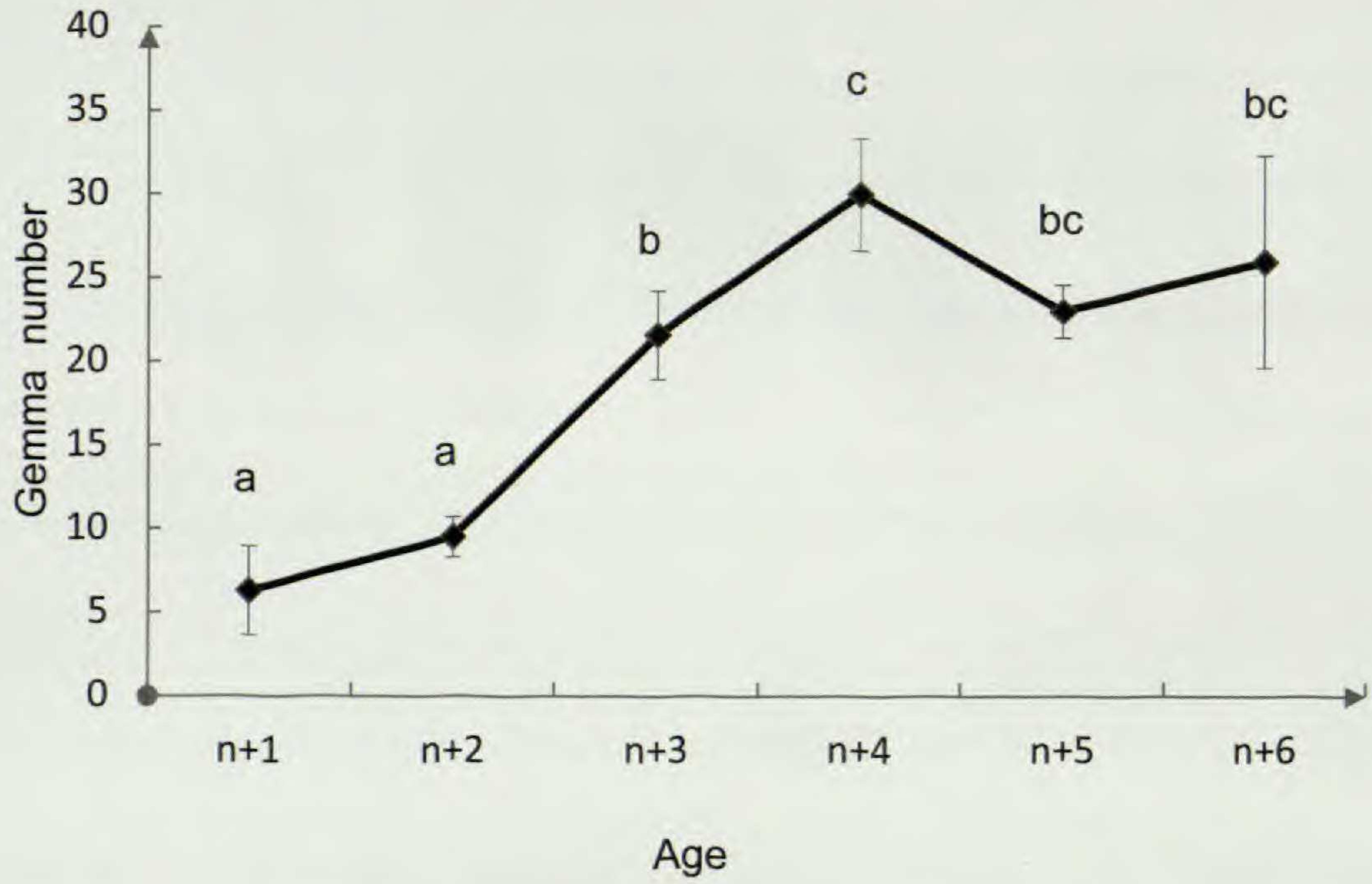

FIG. 3. Gemma numbers of plants at different ages.

Subsequently the leaves in circular shape changed gradually into an ovate shape, and the color of modified gemma leaves at the base of stems faded from green into light yellow. Three months later, the modified gemma leaves became colorless, membranaceous and transparent, then the gemmling was able to live independently. The gemmling height reached about 2 cm after 120 days (Fig. 5). Leaves with serrate margin formed gradually after 120 days.

The effects of different soil matrixes were not significantly different on the gemma gemmation rate, but were significantly different on the seedling survival rate (Fig. 6). For the cultivation in the habitat soil, sand and humus, the average gemmation rate was respectively 95%, 96.7% and 91.7%, with no significant difference ($p>0.05$); the average survival rate of gemmlings was

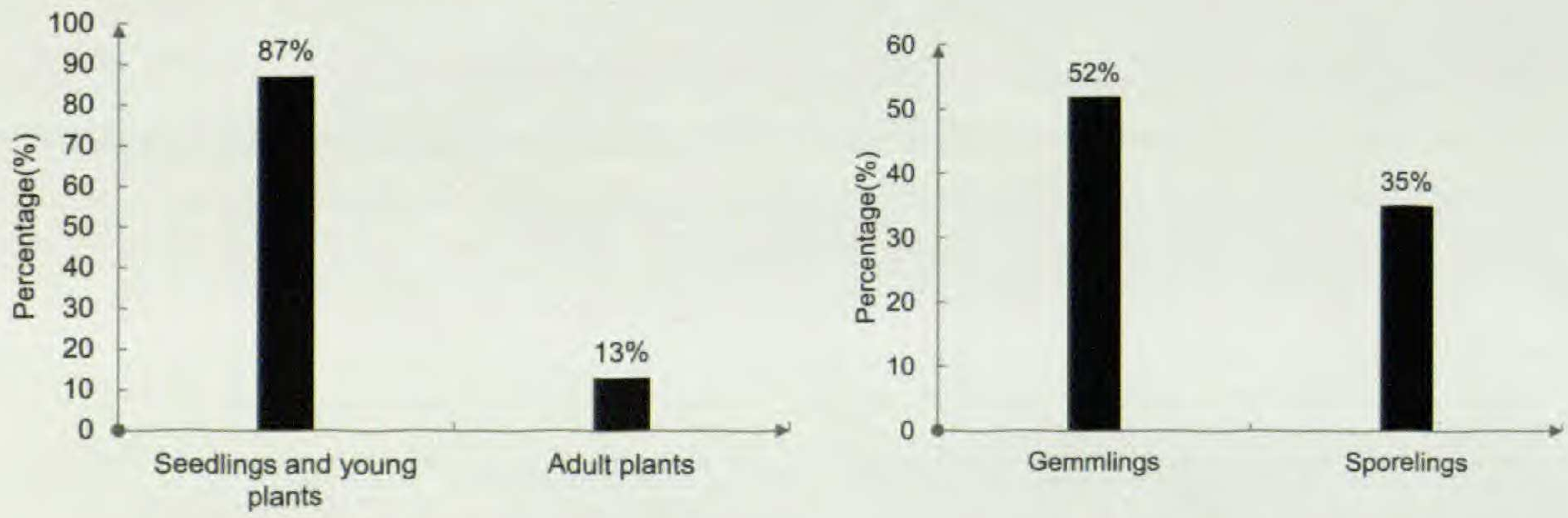

FIG. 4. Population structure: Proportion of adult plants, seedlings (gemmlings and sporelings) and young plants (Left); Proportion of gemmlings and sporelings (Right).

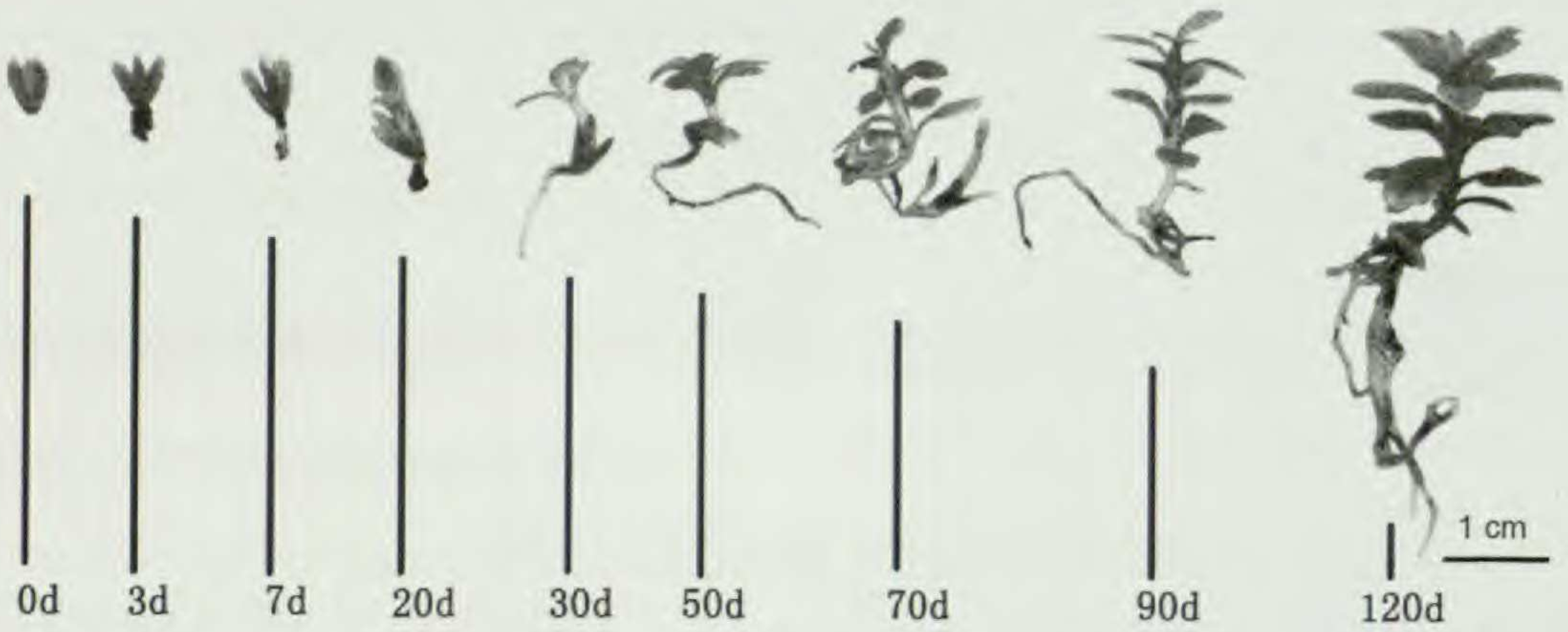

FIG. 5. The gemmlings at different ages.

respectively 91.7%, 48.3% and 88.3%, with the value of the sand medium significantly lower than those of the other two media ($p < 0.01$).

DISCUSSION

The gemma, as a vegetative propagule, has long drawn attention in *Huperzia*. Smith (1920) studied the gemma structure and origin in *Lycopodium lucidulum* Michx., which is included in *Huperzia* now, and he believed that gemmae could germinate into new plants. Reutter (1987) described the growth pattern of *L. lucidulum* from gemma to gemmling. Gola (2008) emphasized again the importance of gemmae in reproductive strategies of *Huperzia* by comparing the gemmae differences of *H. lucidula* and *H. selago* (L.) Bernh. ex Schrank & Mart. between mountain and lowland habitats. Wang *et al.* (2007) studied the gemma structure and the gemmlings of *H. javanica*,

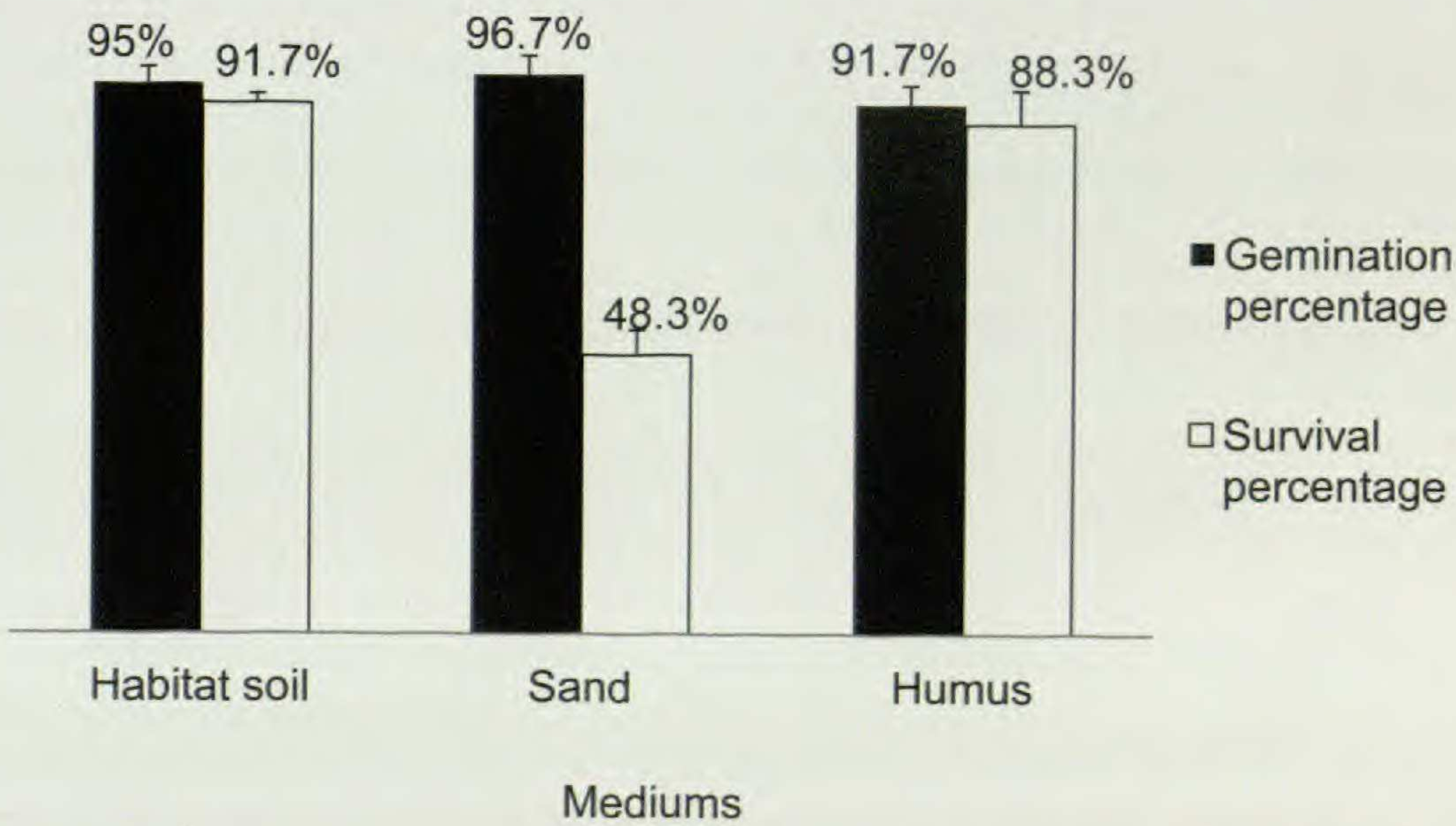

FIG. 6. The percentages of gemmae gemmation and survival of gemmlings in different media of the cultivation experiment.

and noticed that some seedlings developed from gemmae in natural populations. Although most researchers believe that gemmae play an important role in plant dispersal and propagation of *Huperzia*, little is known about the extent to which they act during the regeneration of natural populations.

From 2008 to 2009, we continually observed two growth seasons of a natural population in the Bawangling Nature Reserve of Hainan Province, and surveyed the population structure by field sampling. We found that this was a young population, in which seedlings (gemmlings and sporelings) and young plants accounted for 86.8%, and the gemmlings accounted for 60% of the total seedlings. In gemmlings and sporelings, we deemed that some of plants called sporelings might derive from gemmae because their gemma modified leaves perished. Considering that we did not calculate those gemmlings losing modified leaves at their stem bases, gemmlings could make up a much higher proportion. There was a significant difference between the number of gemmlings and that of sporelings, showing that regeneration of the population mainly depended on gemmae. We also found that the perennial plants of *H. serrata* with over three gemma layers generated more than 20 gemmae per year on average, and that there existed a significant difference between the plants $\geq$ n+3 years old and those <n+3 years old. This shows that the reproductive ability of gemmae became stronger after the third year of gemmae growth.

To further confirm the reproductive ability of the gemmae in *H. serrata*, mature gemmae were collected and used for breeding experiments in habitat soil, sand and humus. These gemmae germinated easily and grew fast, and plant height reached 2 cm in 120 days. The gemmation rate was respectively 95%, 96.7% and 91.7% in the three media, with no significant difference, but the survival rate in sand (48%) was significantly lower than in the other two media (91.7% and 88.3%). Under the cultivation conditions, *H. serrata* gemmae easily germinated using its own stored nutrients, with no special requirements for the cultivation soil. During the gemmling development process, good growth could be expected if the soil provided relatively rich nutrients after the stored nutrients were used. The sand medium did not have enough nutrients to support a high survival of gemmlings, while humus was suitable for gemmling growth as in the habitat soil. This indicates the possibility to quickly establish a cultivation base of *H. serrata* through gemma propagation, thus relieving the resource crisis of *H. serrata*.

Previous studies have showed that *Huperzia* spores take 2–5 years to develop into sporophyte (Bruchmann, 1910; Whittier, 2007). The plants of *H. serrata* grow more slowly, normally requiring 15–20 years of growth from spore germination to maturity (Ma *et al.*, 2006). Up to now, spore germination techniques in soil or in axenic culture have not been established. Despite the success in culturing the shoot of *Huperzia selago* (Szyputa *et al.*, 2005), it has been difficult to culture *H. serrata* shoots because of the endophytic fungi (Shen *et al.*, 2002). The cutting propagation of *H. serrata* has also been reported (Sheng *et al.*, 2000). As stem tips are used as cuttings in this technique, large amounts of wild resources are consumed. Because of the shortage of the species in the wild, it is almost impossible to make large-scale production. Compared with spore collection and cutting, gemmae have the advantages of being abundant, high propagation rate and fast growth. Our results may not only reveal the important role of gemmae in reproductive strategies, but also be helpful to resource protection and cultivation of *H. serrata*.

ACKNOWLEDGMENTS

We thank Jin-Qiang Wang and Zhao-Li Zhou from the Bawangling Nature Reserve in Hainan Province, China for their kind help in field. This work was supported by the Central Public-interest Scientific Institution Basal Research Fund (2007HNB003), Natural Science Foundation of Hainan Province, China (309132) and the National Science & Technology Pillar Program of China (2007BAI27B01).

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